

Galectin-3-Binding Protein: A Serological and Histological Assessment in accordance with Hepatitis C-related Fibrosis.

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Abstract

Objectives. Invasive liver biopsy is the current method for the assessment of liver fibrosis. In search of non-invasive alternatives, galectin-3-binding protein was introduced as a candidate-marker of hepatitis C-related fibrosis based on serum proteomics.

We investigated the role of galectin-3-binding protein as a single-marker of significant fibrosis and cirrhosis by means of serology and histology and studied the role of glycosylation on the expression in hepatitis C- and alcohol-related cirrhosis.

Methods. Sera and available biopsies of hepatitis C patients with various fibrosis-grades and patients with alcohol-related cirrhosis were used for galectin-3-binding protein measurements by enzyme-linked immunosorbent assay and immunohistochemistry, respectively. Glycosylation-effect was analyzed by Western blot. Data was analyzed in accordance to fibrosis.

Results. Galectin-3-binding protein was increased during cirrhosis ($22.7 \pm 10.1 \mu\text{g/mL}$) in contrast to mild ($11.3 \pm 6.4 \mu\text{g/mL}$) and moderate fibrosis ($13.4 \pm 8.3 \mu\text{g/mL}$) ($p < 0.001$; $p = 0.004$, respectively). Receiver operator characteristics curves showed areas under the curve of 0.68 and 0.81 for the detection of respectively significant fibrosis and cirrhosis. Similar findings in hepatic expression were obtained, in which cirrhosis was associated with diffuse, parenchymal expression ($p = 0.002$). The observed difference between hepatitis C- and alcohol-related cirrhosis ($13.5 \pm 9.0 \mu\text{g/mL}$) ($p = 0.009$) could not be explained by glycosylation. (mean \pm standard deviation)

Conclusions. Our recent findings confirm our initial proteome results on serological and histological level as well as the role of galectin-3-binding protein as a marker of

hepatitis C-related fibrosis, especially cirrhosis. Implication of this protein in future multi-marker study should be considered.

Keywords

Galectin-3-binding protein, hepatitis C, liver fibrosis, liver cirrhosis, alcohol, immunohistochemistry, western blot, liver biopsy, ELISA, serum, bio-marker.

1. Introduction

Hepatitis C viral infection is one of the major chronic liver diseases with an estimated prevalence of 3%, about 170 million cases worldwide [1, 2]. Chronic hepatitis C patients are prone to develop liver fibrosis and cirrhosis, characterized by an excessive accumulation of extracellular matrix proteins within the liver [3, 4]. Liver fibrosis is a slow and factor-dependent (i.e. alcohol, age, gender) process and it is therefore essential to closely monitor the fibrosis-progression in order to initialize an appropriate antiviral treatment [3, 5, 6]. Liver biopsy with subsequent histological examination is the current diagnostic standard for fibrosis assessment, but is limited due to invasiveness and potential sampling error; this has stimulated the search for non-invasive alternatives [7-10].

In this regard, we previously analyzed the serum proteome of chronic hepatitis C patients in search of new protein-markers of fibrosis by means of two-dimensional gel electrophoresis and mass spectrometry; Among the identified proteins, galectin-3-binding protein was shown to be specifically up-regulated in advanced stages of fibrosis, especially cirrhosis [11]. Galectin-3-binding protein is a secreted 90 kDa *N*-glycosylated protein with affinity for galectins as well as extracellular matrix proteins [12]. Sudden increase in serum galectin-3-binding protein levels have been reported in various infectious and cancerous diseases, including hepatitis C [13-22].

In the present study, we investigated the role of galectin-3-binding protein as a single-marker of hepatitis C-related fibrosis by means of serological and histological assessment of hepatitis C patients. Furthermore, we investigated the role of glycosylation in the difference of serum galectin-3-binding protein expression in

hepatitis C- and alcohol-related cirrhosis [17, 23]. We chose cirrhosis for this comparison due to substantial difference in fibrogenesis and pathological scoring systems in pre-cirrhotic stages [24].

2. Methods

2.1. Study population

This mono-centric study consisted of chronic hepatitis C patients (n = 76), alcohol-related cirrhotic patients (n = 11) and controls (n = 5), registered at the Department of Gastroenterology and Hepatology at Ghent University Hospital (Ghent, Belgium). All patients gave informed consent prior to the start of the study. The study was also approved by the Ethical Committee of the Ghent University Hospital.

Viral status was confirmed by anti-HCV enzyme immunoassay (EIA), HCV RNA-PCR, while fibrosis-grade (F0 = no fibrosis; F1 = portal fibrosis without septa; F2 = portal fibrosis with few septa; F3 = numerous septa without cirrhosis; F4 = cirrhosis) and inflammatory activity (A0 = no activity; A1 = mild activity; A2 = moderate activity; A3 = severe activity) were assessed by liver biopsy according to the Metavir scoring-system [25, 26].

All patient's sera were stored at -20 °C at the Department of Gastroenterology and Hepatology, while available tissue specimens of chronic hepatitis C patients (n = 20) were obtained from the Department of Pathology at Ghent University Hospital (Ghent, Belgium). Patients with anti-viral treatment, co-infection, severe decompensation according to the Child-Pugh score (> score A) (bilirubin > 2

mg/dL, albumin < 3.5 mg/dL, international normalized ratio > 1.7, ascites and encephalopathy) or hepatocellular carcinoma (HCC) were excluded from the study.

2.2. Serum galectin-3-binding protein measurement

Serum galectin-3-binding protein levels were measured by means of a commercially available enzyme-linked immunosorbent assay (ELISA) (BMS234, Bender MedSystems®, Vienna, Austria). Briefly, patient's sera were diluted and loaded onto a monoclonal antibody-coated 96-microwell plate. The plate was incubated at 37 °C for 45 min, subsequently washed and re-incubated with a second monoclonal horse radish peroxidase (HRP-) labeled antibody at 37 °C for 45 min. After repeating the washing procedure, tetramethylbenzidine (TMB) substrate solution was added to the wells and incubated at room temperature for 15 min while shaking at 100 rpm. Substrate reaction was stopped after addition of an acid-based stop solution. Absorbance was measured and analyzed by UV-VIS spectrophotometry (Safire², Tecan, Männedorf, Switzerland) at 450 nm; serum galectin-3-binding protein levels were determined by plotting the standard curve. The intra- and inter-assay coefficients of variation were 5.5 and 11.9%, respectively, according to user's manual.

2.3. Galectin-3-binding protein immunohistochemistry

Paraffin-embedded sections (5 µm) were dewaxed in an iso-paraffin based clearing agent (UltraClear®, Klinipath, Geel, Belgium) and rehydrated in graded ethanol baths (99% and 96%) (Klinipath). Antigen retrieval was performed with citrate buffer, containing 10 mM sodium citrate (pH 6) (Sigma-Aldrich, Steinheim,

Germany) and 0.05% Tween-20 (MP Biomedicals, Solon, OH, USA) at 95 °C for 20 min. Non-specific sites on the slides were blocked with phosphate buffer saline (PBS) (pH 7.4) (Invitrogen, Grand Island, NY, USA) containing 1% (w/v) bovine serum albumin (BSA) (Sigma-Aldrich) and 0.3% (v/v) Triton-X (Sigma-Aldrich) for 10 min. Subsequently, tissue sections were incubated with primary antibody (1:200) (R&D Systems, Oxfordshire, UK) overnight at 4 °C. Endogenous peroxidase-activity was blocked with PBS (pH 7.4) (Invitrogen) containing 3% (v/v) H₂O₂ and 1% (w/v) sodiumazide (NaN₃) (Sigma-Aldrich) at room temperature for 20 min. After subsequent incubation in biotinylated secondary antibody and streptavidin-HRP (LSAB + system-HRP, Dako, Carpinteria, CA, USA), galectin-3-binding protein expression was visualized by diaminobenzidine (DAB) (DAB Substrate-Chromogen System, Dako) and haematoxylin-mayer (J.T. Baker, Deventer, Holland). Sections were eventually dehydrated in 96% and 99% ethanol (Klinipath) and iso-paraffine based solution (Klinipath). Primary antibody was replaced by normal goat immunoglobulin G (1:100) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) as negative control.

Staining intensity was blindly assessed by a single pathologist (L.L.) without previous knowledge of the patient's condition at the moment of scoring. Protein stain in the liver parenchyma was either absent (0), centrolobular (1) or panlobular (3) [14].

2.4. Deglycosylation of serum galectin-3-binding protein

Galectin-3-binding protein has about 7 *N*-glycosylation sites, which have substantial influence on antibody affinity; moreover, it is known that serum glycoproteins are

altered in alcoholic and cirrhotic patients [23, 27, 28]. In this regard, the role of *N*-glycans in serum galectin-3-binding protein expression was studied by comparison of deglycosylated galectin-3-binding protein expression in hepatitis C- and alcohol-related cirrhosis.

Denatured serum proteins (1 µg/µL) was prepared by sample dilution in 20 mM ammoniumbicarbonate (pH 8.0) (Sigma-Aldrich) and subsequent denaturation in 0.2% sodium dodecyl sulphate (SDS) (MP Biomedicals) and 10 mM β-mercaptoethanol (Sigma-Aldrich) at 100 °C for 10 min. 1 µL peptide:*N*-glycosidase F (0.5 IUB mU PNGaseF) (Sigma-Aldrich) was added to the samples and incubated at 37 °C for 3 hrs. The enzymatic reaction was stopped by heating up to 95 °C for 5 min in non-reducing sample buffer (Thermo Scientific, Rockford, IL, USA) with 5% β-mercaptoethanol (Sigma-Aldrich). Simultaneously, raw serum proteins (15 µg) were reduced in Laemmli-buffer at 95 °C for 5 min. Laemmli-buffer contained 50 mM TrisHCl (pH 6.8) (MP Biomedicals), 2% (w/v) SDS (MP Biomedicals), 3 M urea (Sigma-Aldrich), 10% (v/v) glycerol (MP Biomedicals) and 5% (v/v) β-mercaptoethanol (Sigma-Aldrich). Samples were resolved on a 10% SDS-polyacrylamide gel in an electrophoresis cell (Criterion™ Cell, Bio-Rad, Hercules, CA, USA) at 150 V and 200 V for 30 min and 1 hr 30 min, respectively. The resolving gel contained 40% acrylamide:bisacrylamide (29:1) (Bio-Rad), 1.5 TrisHCl (pH 8.8) (MP Biomedicals), 10% (w/v) SDS (MP Biomedicals), 10% (w/v) ammonium persulphate (Sigma-Aldrich) and 0.05% (v/v) TEMED (Sigma-Aldrich). Running buffer contained 2.5 mM Tris (MP Biomedicals), 0.1% (w/v) SDS (MP Biomedicals) and 192 mM glycine (Fisher Scientific, Fair Lawn, NJ, USA). Protein transfer onto the nitrocellulose membrane was performed by tank-blotting (Trans-

Blot[®] Electrophoretic Transfer Cell, Bio-Rad) containing transfer solution at 110 V for 90 min. Transfer solution contained 48 mM Tris (MP Biomedicals), 39 mM glycine (Fisher Scientific), 20% (v/v) methanol (VWR International, Leuven, Belgium).

Protein transfer and loading were checked using 0.1% (w/v) Ponceau-S (Sigma-Aldrich) in 5% (v/v) acetic acid (Merck, Hohenbrunn, Germany). Next, the blot was blocked with PBS (pH 7.4) (Invitrogen) containing 1% (w/v) BSA (Sigma-Aldrich) and 0.1% (v/v) Tween-20 (MP Biomedicals) for 1 hr. Subsequently, the blot was incubated with primary antibody (1:400) (R&D Systems) overnight at 4 °C. The blot was washed and incubated with a secondary HRP-labelled bovine anti-goat antibody (1:10000) (Santa Cruz Biotechnology) for 1 hr. After repeating the wash steps, primary antibody was detected by chemiluminescence using luminol/H₂O₂ (SuperSignal[®] West Dura, Thermo Scientific) and imaging System (Versadoc, Bio-Rad). Protein band patterns were analyzed using appropriate software (Quantity One, Bio-Rad).

2.5. Statistical analysis.

Fibrosis-grades according to the Metavir scoring-system was regrouped into three consecutive stages: mild fibrosis (F0 – F1), moderate fibrosis (F2 – F3) and cirrhosis (F4). Inflammatory activity-grading was regrouped into two stages: mild activity (A0 – A1) and significant activity (A2 – A3). Genotypes were regrouped into genotype 1 – 4, 2 – 3 and others according to previous findings, suggesting a more prominent increase of serum galectin-3-binding protein in genotype 1 and 4 [13, 14, 16, 19].

Continuous data and categorical/nominal data were analyzed in accordance with fibrosis-grades by means of respectively analysis of variances (ANOVA) and Fisher's exact test. Data was log-transformed if appropriate.

Receiver operator characteristic (ROC) curves and area under the curve (\pm standard error; 95% confidence interval) were calculated in order to evaluate the performance of serum galectin-3-binding protein as single-marker in the detection of significant fibrosis (\leq F2) and cirrhosis (F4). Statistical analyses were performed in SPSS v.15.0 (SPSS, Chicago, IL, USA) and R v.2.8.1 (R-software). A p-value < 0.05 was considered statistically significant.

3. Results

3.1. Serum galectin-3-binding protein

Serum galectin-3-binding protein expression was associated with hepatitis C-related fibrosis-grades ($p < 0.001$), similar to age at sampling and albumin amount (Table 1). Post-hoc analysis even showed an increase during cirrhosis (F4) (22.7 ± 10.1 $\mu\text{g/mL}$) in contrast to pre-cirrhotic stages (F0 – F1, F2 – F3) ($p < 0.001$ and $p = 0.004$, respectively). Despite significant distinction between fibrosis-grades, albumin-, bilirubin- and INR-values did not drastically exceed normal reference-values (Table 1).

ROC-curves showed that serum galectin-3-binding protein has an area under the curve of $0.68 (\pm 0.06; [0.56 - 0.80])$ and $0.81 (\pm 0.06; [0.69 - 0.91])$ for the detection of respectively significant fibrosis (\leq F2) and cirrhosis (F4) (Figure 1).

Serum levels above 12.9 $\mu\text{g/mL}$ have a sensitivity of 0.60 and specificity of 0.65 for the diagnosis of significant fibrosis; while levels above 16.9 $\mu\text{g/mL}$ have a sensitivity of 0.70 and specificity of 0.75 for the diagnosis of cirrhosis (Figure 1).

3.2. Hepatic galectin-3-binding protein

Immunohistochemistry of hepatitis C-related specimens showed positive, granular staining within cytoplasm of hepatocytes and occasional staining of reactive bile ductules and bile canaliculi (Figure 2).

Statistical outcomes of the biopsy-population were strongly similar to the total hepatitis C population of this mono-centric study (Table 2). Positive hepatocellular expression was shown to be correlated with fibrosis-grades and steatosis ($p = 0.002$; $p = 0.007$, respectively) and was independent of patient's gender, viral genotype and inflammatory activity. Analysis of the histological expression in association with fibrosis-grade resulted in the following characteristics:

F0 – F1 was characterized by weak and moderate, centrolobular expression, F2 – F3 was characterized by moderate, centrilobular and diffuse, panlobular expression, while F4 was associated with diffuse, panlobular expression (Figure 2). The difference in histological expression between fibrosis-grades was significant between F0 – F1 and F4 following post-hoc analysis ($p < 0.05$).

Moreover, serum galectin-3-binding protein levels and immunohistochemistry were significantly associated with each other ($p = 0.038$); diffuse, panlobular expression was associated with a mean galectin-3-binding protein concentration of 25.1 (± 9.9) $\mu\text{g/mL}$.

3.3. Hepatitis C- opposite alcohol-related cirrhosis

In general, cirrhosis is similar in all chronic liver diseases being characterized by an overall loss of normal liver structure due to regenerative nodule-formation. In addition, histological expression in alcoholic liver specimen has shown a similar diffuse staining pattern (Figure 2).

Both pathologies showed a significant increase in contrast to healthy controls ($3.2 \pm 1.3 \mu\text{g/mL}$) ($p < 0.001$); serum galectin-3-binding protein levels were $13.5 (\pm 9.0) \mu\text{g/mL}$ and $22.7 (\pm 10.1) \mu\text{g/mL}$ for respectively alcohol- and hepatitis C-related cirrhosis (Figure 3). Moreover, serum levels in hepatitis C-related cirrhosis were more elevated than in alcoholic cirrhosis ($p = 0.009$). Alcoholic patients were not severely decompensated at the time of sampling (data not shown).

In order to investigate whether glycosylation is involved in this observed discrepancy, a qualitative study of deglycosylated galectin-3-binding protein was conducted by means of Western blotting. Untreated serum galectin-3-binding protein band was observed at $\pm 100 \text{ kDa}$ following Western blot, while deglycosylation resulted in two minor bands at 60 kDa due to the loss of all *N*-glycans (Figure 3). No difference was detected in band expression of untreated and deglycosylated galectin-3-binding protein between both aetiologies.

Discussion

Non-invasive assessment of liver fibrosis in chronic hepatitis C (CHC) patients has evolved rapidly in the past ten years due to new pathological insights and technologies;

diagnostic techniques, such as the Fibroscan, have already been implemented into daily practice [29, 30]. Technological progress also enables new ways to discover useful disease markers (i.e. proteomics, glycomics) [11, 31-33]. Our previous serum proteome study in hepatitis C patients identified galectin-3-binding protein as marker of advanced fibrosis, especially cirrhosis [11].

In the current study, we investigated the role of this protein as a single-marker of significant hepatitis C-related fibrosis and cirrhosis by means of serological and histological testing. Serum measurement and subsequent univariate analysis confirmed our previous proteome findings by detecting a gradual increase in protein level towards end-stage liver disease. Like most single-marker studies, clear distinction was observed between the two most extreme stages (F0 – F1 and F4) with substantial overlap between the other stages. Serum galectin-3-binding protein showed a good diagnostic performance for the detection of cirrhosis, in which optimal sensitivity and specificity were similar for the respectively detection and exclusion of cirrhosis (about 0.70).

In contrast to many previous marker reports, we confirmed our proteome findings by detecting galectin-3-binding protein within liver tissue of chronic hepatitis C patients with varying fibrosis-grades. Moreover, despite the limited sample size, we were able to confirm a same increase in protein-level at hepatic tissue level. A clear distinction in hepatocellular expression was observed between mild fibrosis (F0 – F1) and cirrhosis (F4), which was characterized by a clear zonular expression in centro- and panlobular areas. Serological and histological outcome were even positively correlated following association-studies. The expression of galectin-3-binding protein was also accompanied by hepatic steatosis, a common feature of chronic hepatitis C; previous reports have associated steatosis with fibrosis progression and severity [34-39].

Besides hepatocellular expression, occasional galectin-3-binding protein staining was detected in reactive bile ductules and canaculi, which has not been previously reported. Bile ductular reaction is known to increase during hepatitis C-related cirrhosis development due to bile ductular metaplasia [40, 41]; this might imply a potential role for galectin-3-binding protein in very early stages of neoplasia or abnormal cellular manifestations. Galectin-3-binding protein has already been associated with the bile proteome as well as biliary tract carcinoma [20, 42]. On the other hand, detection in canaliculi confirms an active protein secretion by hepatocytes into the extracellular space.

Difference in serum levels of galectin-3-binding protein in cirrhotic patients due to etiology has previously been observed [17]; in this regard, we investigated whether post-translational glycosylation has an intrinsic effect on protein expression in hepatitis C- and alcohol-related cirrhosis. However, this was not the case, suggesting a more *in vivo* alteration in gene or protein regulation due to difference in molecular pathway [3, 4, 43-47]. Evidence of these alterations in hepatitis C has been provided by Bigger *et al.*, reporting a change in hepatic gene regulation during course and clearance of viral hepatitis C in primates [48]. Also, previous studies have associated increased serum galectin-3-binding protein levels with viral genotype 1 - 4 [13, 14, 16, 19]. According to these findings, galectin-3-binding protein is likely a hepatitis C-specific marker of fibrosis rather than a universal fibrosis marker, despite significant increase in protein-level in both pathologies.

Galectin-3-binding protein is believed to have immuno-, cytokine modulating properties, but the actual functional role in viral hepatitis C and liver fibrosis remains unclear [49, 50]. On the other hand, galectin-3 has been associated with active

regulation of fibrosis [51, 52]. The absence of galectin-3-binding protein stain at the actual fibrotic lesions as well as lack of correlation with inflammation in our study, suggest a possible indirect involvement in hepatitis C-related liver fibrosis [53]. In addition, the increase in protein levels in sera and tissue could also be an indication of early neoplastic events [17, 20].

In conclusion, we provided confirmation of our proteome study and substantial evidence that galectin-3-binding protein expression is representative for the progressive, aggravating events of the liver following viral hepatitis C at a serological as well as histological level. At a single-marker level serum galectin-3-binding protein shows potential as an indicator of compensated cirrhosis; it might therefore be interesting to integrate this serum protein in a multi-marker test for the detection of hepatitis C-related liver fibrosis. Our results warrant the comparison of the accuracy of this marker with that of other proposed non-invasive markers of fibrosis [54-57].

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Table 1. Hepatitis C patient's characteristics (n = 76).

	Valid cases*	F0 – F1 (n = 26)	F2 – F3 (n = 27)	F4 (n = 23)	p-value [†]
Gender (male/female)	100%	14/12	15/12	15/8	0.71
Genotype (1-4/2-3/other)	93%	16/5/3	21/4/1	18/3/0	0.50
Activity (A0-A1/A2-A3)	83%	23/1	15/12	6/6	< 0.001
Age (years)	100%	39.7 (± 13.1)	50.6 (± 14.7)	58.0 (± 11.0)	< 0.001
BMI (kg/m²)	80%	24.0 (± 3.8)	25.0 (± 4.4)	26.6 (± 3.2)	0.11
G3BP (µg/mL)[‡]	100%	11.3 (± 6.4)	13.4 (± 8.3)	22.7 (± 10.0)	< 0.001
Albumin (3.4 – 4.8 g/dL)	99%	4.6 (± 0.3)	4.5 (± 0.3)	4.0 (± 0.3)	< 0.001
Bilirubin (0.3 – 1.2 mg/dL)[‡]	99%	0.7 (± 0.5)	0.7 (± 0.4)	1.1 (± 0.9)	0.006
INR (0.9 – 1.3)	99%	1.0 (± 0.1)	1.0 (± 0.1)	1.3 (± 0.1)	0.02

Body mass index (BMI), galectin-3-binding protein (G3BP), International normalized ratio (INR).

*Valid cases represent the percentage of available data.

Numeric data is represented in mean (± standard deviation) and categorical/nominal data in counts.

[†]parameters were analyzed in accordance with fibrosis-grades by means of univariate analysis of variances (ANOVA) and Fisher's exact test for respectively continuous and categorical/nominal data.

[‡]Statistical analysis conducted on log-transformed data.

Table 2. Hepatitis C patient's characteristics of the liver biopsies (n = 20).

	Valid cases*	F0 – F1 (n = 7)	F2 – F3 (n = 9)	F4 (n = 4)	p-value [†]
Gender (male/female)	100%	¾	4/5	3/1	0.71
Genotype (1-4/2-3/other)	95%	6/0/0	8/0/1	3/1/0	0.47
Activity (A0-A1/A2-A3)	83%	7/0	3/6	2/2	0.02
Steatosis (absent/present)	100%	7/0	5/4	0/4	0.003
G3BP (E1/E2/E3)*	100%	3/0/0	4/6/0	0/3/4	0.002
Age (years)	100%	32.9 (± 14.4)	54.4 (± 14.5)	45.3 (± 12.7)	0.03
BMI (kg/m²)	80%	24.2 (± 3.8)	26.4 (± 5.0)	27.9 (± 2.3)	0.43
Albumin (3.4 – 4.8 g/dL)	99%	4.7 (± 0.1)	4.4 (± 0.2)	4.0 (± 0.3)	0.001
Bilirubin (0.3 – 1.2 mg/dL)[‡]	99%	0.9 (± 0.9)	0.7 (± 0.4)	1.4 (± 1.5)	0.64
INR (0.9 – 1.1)	99%	1.0 (± 0.1)	1.0 (± 0.1)	1.2 (± 0.2)	0.04

Body mass index (BMI), galectin-3-binding protein (G3BP), International normalized ratio (INR), expression-pattern (E): weak (E1), moderate (E2) and diffuse (E3).

*Valid cases represent the percentage of available data.

Numeric data is represented in mean (± standard deviation) and categorical/nominal data in counts.

[†]parameters were analyzed in accordance with fibrosis-grades by means of univariate analysis of variances (ANOVA) and Fisher's exact test for respectively continuous and categorical/nominal data.

[‡]Statistical analysis conducted on log-transformed data.

Figure legends

Figure 1. Receiver operator characteristic (ROC) curves with corresponding area under the curve-values (0.68 and 0.81) of serum galectin-3-binding protein for the detection of respectively significant fibrosis (\geq F2) (a) and cirrhosis (F4) (b). Dot-plot (c) shows the individual serum measurements of galectin-3-binding protein: cut-off value at 12.9 μ g/mL (full line), galectin-3-binding protein has sensitivity of 0.60 and specificity of 0.65 in the detection of significant fibrosis (\geq F2); while cut -off value at 16.9 μ g/mL (dashed line), galectin-3-binding protein has a sensitivity of 0.70 and specificity of 0.75 for the detection of cirrhosis (F4).

Figure 2. Galectin-3-binding protein expression in hepatitis C-related (a – c) (20x magnification) and alcoholic cirrhosis (d) (40x magnification) by means of immunohistochemistry and haematoxylin background-staining. Positive immuno-stain was represented by a brown-like stain within cytoplasm of the hepatocytes (a – d), while negative staining was represented by a blue-like stain, seen in the negative control (n.c.). Three different positive staining-patterns could be distinguished: no or rare positive hepatocytes (a); cytoplasmic positivity in the centrolobular hepatocytes (b); diffuse, panlobular staining of the parenchyma (c). In addition, occasional staining of reactive bile ductules (BD) and caniculi (CA) were detected (d). Vascular structures (a) (black arrow) and mononuclear infiltrates (c) (white arrow) were negatively stained. Central vein (CV) and portal tract (PT) (b).

Figure 3. Serum galectin-3-binding protein levels in healthy controls, hepatitis C-related and alcoholic cirrhosis by means of enzyme-linked immunosorbent assay (ELISA) (a) and Western blot (b). (a) Increase in serum levels was observed in cirrhotic patients in contrast to healthy controls ($p < 0.001$); moreover, serum levels in hepatitis C-related cirrhosis were more elevated in contrast to alcoholic cirrhosis ($p = 0.009$). error bar graph represent mean-values with bars representing standard error of the mean. (b) protein bands were detected at ± 100 kDa (raw) (3 and 5) and ± 60 kDa (deglycosylated) (4 and 6). The minor bands (arrows) after deglycosylation showed no significant difference in expression or pattern between alcohol- and hepatitis C-related cirrhosis. Galectin-3-binding protein in the healthy control (lane 1 and 2) was below detection limit.